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(54) Title: PROCESS FOR SIALYLATING GLYCOPROTEINS

(57) Abstract

The present invention relates to genetic engineering, and provides a novel process for sialylating glycoproteins. In the process said proteins are incubated with recombinant yeast cells expressing a sialyltransferase enzyme as a fusion protein with Hsp150Δ carrier in the porous cell wall.

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Process for sialylating glycoproteins

Field of the invention

The present invention relates to genetic engineering, and provides a novel and efficient process for sialylating glycoproteins. In the process said proteins are incubated with recombinant yeast cells expressing a sialyltransferase enzyme as a fusion protein with Hsp150Δ carrier in the porous cell wall.

Background of the invention

Most pharmaceutical proteins desired to be manufactured in cultured cells by genetic engineering are secretory proteins, which carry sialylated N-glycans in their authentic form. The host cell, as well as cell culture conditions, have profound effects on glycosylation of the protein product (Andersen and Goochee, 1994; James *et al.*, 1995). The host cell may lack sialyltransferases, like yeast, fungal and insect cells, or shed sialidase activity which desialylates the protein product after secretion (Gramer *et al.*, 1995). The sialylation of recombinant mammalian proteins performed by mammalian cells is often incomplete, or not authentic (Curling *et al.*, 1990; Goochee *et al.*, 1991; Patel *et al.*, 1992; Maiorella *et al.*, 1993). For instance, cultured CHO and BHK-21 cells lack a functional α2,6-sialyltransferase and add only α2,3-sialic acid to glycoproteins, whereas human and mouse cells decorate glycoproteins with both residues (Lee *et al.*, 1989). Lack of terminal sialic acid residues may or may not affect the biological activity of the protein, but the clearance rate from the blood stream is dramatically increased. Proteins with exposed terminal galactose residues are removed from the circulation, severely compromising the effect of the pharmaceutical proteins (Fukuda *et al.*, 1989; Sareneva *et al.*, 1993; Szkudlinski *et al.*, 1993).

Authentic sialyltransferases are type II transmembrane glycoproteins anchored to the Golgi membrane. The catalytic ectodomain faces the Golgi lumen and a short N-terminal segment the cytoplasm (Field and Wainwright, 1995) (see

Fig. 1). We have constructed a *Saccharomyces cerevisiae* strain, which expresses the ectodomain of rat liver α2,3-sialyltransferase ($ST3N_e$) as a fusion protein (Mattila *et al.*, 1996). Glycosyltransferases like many mammalian secretory proteins are retained in the endoplasmic reticulum (ER) in yeast (Krezdorn *et al.*, 1994). Thus we linked $ST3N_e$ to the C-terminus of the Hsp150Δ polypeptide, which consists of the 321 N-terminal amino acids of the natural secretory glycoprotein Hsp150 of yeast (Russo *et al.*, 1992). The Hsp150Δ polypeptide has been shown to promote proper folding and secretion competence of several foreign proteins in *S. cerevisiae* (Simonen *et al.*, 1994 and 1996; Makarow, US patent No. 5,677,172). The 162 N-terminal amino acids of the Hsp150 protein also promote folding of foreign proteins and confer them secretion competence. Thus the Hsp150Δ carrier is defined as an N-terminal fragment of the translation product of the *HSP150* gene, having 162 to 321 amino acid residues. The Hsp150Δ-$ST3N_e$ fusion protein was efficiently secreted in enzymatically active form, but adhered tightly to the yeast cell wall (see Fig. 1). Incubation of the recombinant yeast cells with N-acetyllactosamine and CMP-Neu5Ac resulted in α2,3-sialylation of the disaccharide, demonstrating that the substrates as well as the product penetrated the cell wall efficiently (Mattila *et al.*, 1996). Previously, heterologous proteins have been immobilized in the yeast cell wall e.g. by a fragment of the yeast a-agglutinin protein (Schreuder *et al.*, 1993), whereas in here, the fusion protein as such is bound to the cell wall.

Summary of the invention

In this invention we show that, surprisingly, also whole glycoproteins can penetrate easily the yeast cell wall and reach the ST3N activity. Several desialylated mammalian glycoproteins were shown to be sialylated by incubating them with the whole living recombinant yeast cells, which express a sialyltransferase enzyme as a fusion protein with Hsp150Δ carrier in the porous cell wall.

In this work whole *Saccharomyces cerevisiae* cells expressing the catalytic ectodomain of rat liver α2,3-sialyltransferase ($ST3N_e$) in the porous cell wall were used to complement sialylation of glycoproteins with native conformations. Incubation of the yeast cells with desialylated fetuin, prothrombin and transferrin resulted in transfer of Neu5Ac from CMP-Neu5Ac to the terminal galactose residues of the protein-bound N-glycans. The K_m values of the yeast cell wall-borne enzyme for CMP-Neu5Ac, N-acetyllactosamine and lacto-N-tetraose were similar to those of recombinant $ST3N_e$ produced in insect cells and of authentic rat liver ST3N. The recombinant yeast strain provides an inexpensive and self-perpetuating source of ST3N activity for sialylation of glycoproteins.

Detailed description of the invention

In the following the process of the invention is illustrated by the experimental procedure of the sialylation of bovine plasma fetuin, bovine prothrombin and human transferrin by incubating said proteins with CMP-Neu5Ac and whole living recombinant *S. cerevisiae* cells expressing the catalytic ectodomain of rat liver α2,3-sialyltransferase ($ST3N_e$).

Brief description of the drawings

Figure 1. Authentic sialyltransferases are located in the Golgi membrane (GM). The catalytic ectodomain faces the lumen and a short N-terminal sequence the cytoplasm (CP). We expressed the ST3N catalytic ectodomain in yeast cells as a fusion protein, its N-terminus linked to the C-terminus of the Hsp150Δ polypeptide (wavy line). In yeast cells the fusion protein is transported to the exterior of the yeast plasma membrane (PM), but remains bound to the cell wall (CW).

Figure 2. Transfer of [^{14}C]Neu5Ac to asialofetuin and asialomucin by whole yeast cells expressing Hsp150Δ-$ST3N_e$ in the cell wall. **(A)** Duplicate samples of yeast strain H626 were incubated for 1-4 hours with 100000 cpm of CMP-

[^{14}C]Neu5Ac and asialofetuin (closed circles), with completely sialylated fetuin (open circles), or with asialomucin (diamonds). Parental yeast cells (H23) were incubated with asialofetuin (open squares). All incubations were in the presence of 10 mM NaN_3. **(B)** Asialofetuin was incubated as above with NaN_3 (closed circles), or with 40 mM DTT (closed squares), or with 4% glucose (open circles), or with 4% glucose and 40 mM DTT (open squares). TCA-precipitated radioactivity is plotted against incubation time.

Figure 3. MALDI-TOF mass Spectra of the N-glycans. Oligosaccharides liberated from asialofetuin (A), or from asialofetuin incubated for 4 h (B) or 8 h (C) with H626 cells. In **(A)**, signals were assigned to nonsialylated triantennary glycans (m/z 2008, $[M+H]^+$; m/z 2030, $[M+Na]^+$) and nonsialylated biantennary glycans (m/z 1665, $[M+Na]^+$). In **(B)** and **(C)**, monosialylated biantennary (m/z 1956, $[M+Na]^+$; m/z 1978, $[M-H+2Na]^+$); monosialylated triantennary (m/z 2321, $[M+Na]^+$; m/z 2343, $[M-H+2Na]^+$); disialylated triantennary (m/z 2612, $[M+Na]^+$; m/z 2634, $[M-H+2Na]^+$; m/z 2656, $[M-H+3Na]^+$) and trisialylated triantennary glycans (m/z 2903, $[M+Na]^+$; m/z 2925, $[M-H+2Na]^+$) were detected. The asterisk (*) designates matrix adducts typical to this mode of analysis (Nyman *et al.*, 1998).

Materials and methods

Strains and media. *S. cerevisiae* strains H23 (Mat*a* *his3-11, 15 leu2-3,112 trp 1-1 ade2-1 can1-100 hsp150::URA3*) and H626 (Mat*a* *his3-11,15 leu2-3,112 ade2-1 can1-100 hsp150::URA3 TRP1::HSP150Δ-ST3N$_e$*) (Mattila *et al.*, 1996) were grown at 24°C in YPD medium consisting of 1% yeast extract (Oxoid Ltd., UK), 2% bacto peptone (Difco, Detroit, MI) and 2% glucose (BDH Pharmaceuticals Ltd., UK), or in synthetic complete (SC) medium (Simonen *et al.*, 1994) lacking tryptophane for selection.

Sialyltransferase assays. Duplicate samples of 5 x 10^7 whole living yeast cells were incubated in 70 µl of 50 mM imidazole buffer, pH 7 (glycoproteins), or of 50 mM Tris-maleate buffer, pH 6.7 (oligosaccharides), either with 0.2

nmol (100000 cpm) of CMP-[^{14}C]Neu5Ac (294 mCi/mmol, Amersham International, Buckinghamshire, UK) or with saturating concentrations of unlabelled CMP-Neu5Ac (Sigma, St. Louis, MO), and varying amounts of the acceptor substrates, in a shaker at 24°C. For determination of intracellular plus extracellular ST3N activity, 5 x 10^7 cells were lysed mechanically with glass beads (Mattila *et al.*, 1996) prior to the sialyltransferase assay. Recombinant $ST3N_e$ (0.34 mU) produced in Sf9 cells (Calbiochem-Novabiochem, La Jolla, CA) was incubated at 37°C in 20 µl of 50 mM MOPS buffer, pH 7.5, containing 1% BSA, and 0.06 nmol of CMP-[^{14}C]Neu5Ac (28500 cpm) or saturating concentrations of unlabelled CMP-Neu5Ac, and different amounts of the acceptor substrate. After sialylation, the proteins were precipitated with 20% TCA for 30 min on ice and collected on filters for scintillation counting. Oligosaccharides were applied on columns of Dowex AG 1 (acetate form, BioRad, Hercules, CA) and Dowex 50 (H^+form, Fluka, Switzerland). Neutral oligosaccharides were eluted with 4 ml of water and sialyloligosaccharides with 20 ml of 0.5 M acetic acid (Renkonen *et al.*, 1991), and subjected to scintillation counting. Fetuin, asialofetuin, asialoprothrombin, asialomucin, lacto-N-tetraose and N-acetyllactosamine were from Sigma, St. Louis, MO, transferrin from the Finnish Red Cross, Helsinki, Finland, and prothrombin from ICN, Aurora, Ohio. Desialylation of prothrombin and transferrin was performed in 0.025 M H_2SO_4 for 1 h at 80°C (Spiro, 1960), whereafter the preparations were neutralized and ultrafiltrated using Centricon devices (cut off 30 kD) to remove the released Neu5Ac residues and the ions.

Isolation of N-glycans. After sialylation, the proteins were desalted on reversed-phase HPLC and the fractions dried in a vacuum centrifuge. The samples were dissolved in 10 µl of 20 mM sodium phosphate, pH 7.2, containing 1% SDS, and boiled for 3 minutes. After cooling, 75 µl of 20 mM sodium phosphate, pH 7.2, 10 µl of 10% OGP and 1 U of N-glycosidase F (Boehringer Mannheim GmbH, Germany) were added and the mixtures were incubated at 37°C for 3 days. Proteins and detergents were removed using a BondElut® C18-column (Analytichem International, CA, USA). The samples were diluted to 300 µl with water prior to loading onto the column, and the glycans were

eluted with 1.5 ml of water. Buffer salts were removed by drop-dialysis against water on VSWP 02500 membranes (Millipore, Bedford, MA, USA) (Börnsen *et al.*, 1995).

Mass spectrometry and CD spectroscopy. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a Biflex™ instrument (Bruker-Franzen Analytik, Bremen, Germany) equipped with a nitrogen laser operating at 337 nm. N-glycans were analyzed in the linear positive ion delayed extraction mode by using 2,4,6-trihydroxyacetophenone (THAP, 3 mg/ml in acetonitrile/20 mM aqueous diammonium citrate, 1:1) as matrix. Samples were prepared by mixing 0.5 µl of drop-dialysed oligosaccharide solution (see isolation of N-glycans) and 0.5 µl of THAP matrix on the target plate, and immediately dried under vacuum to produce a thin homogenous matrix (Papac *et al.*, 1996). This mode of analysis causes little loss of Neu5Ac residues, and may be used for rapid quantitation of both sialylated and neutral oligosaccharides in a mixture (Nyman *et al.*, 1998). The mass spectra were externally calibrated with Dextran 5000 (Fluka BioChemica, Switzerland). CD spectroscopy was performed using a JASCO J-720 spectropolarimeter. The spectra were recorded in the far UV region (190-259 nm). Each spectrum was the mean of five scans obtained with a time constant of 1 s and a speed of 20 nm/min.

Kinetic studies. The K_m determinations were according to Lineweaver-Burke. To determine the activity of Hsp150Δ-ST3N$_e$, H626 cells (5 x 10^7) were incubated with 0.4 mM lacto-N-tetraose and 10 mM CMP-Neu5Ac. The supernatant was subjected to ion exchange chromatography over the Dowex columns and the eluates were concentrated and chromatographed over a Superdex® Peptide PC 3.2/30 column (Pharmacia, Sweden) (Nyman *et al.*, 1998). The oligosaccharides were quantitated against external lacto-N-tetraose (Sigma) and NeuNAc (Sigma).

Results

Transfer of [^{14}C]Neu5Ac to N-glycans of asialoglycoproteins.

Authentic bovine plasma fetuin (M_w 48 kD) carries three N-glycans (Spiro, 1973; Rice *et al.*, 1990). To study whether asialofetuin could penetrate the yeast cell wall and be sialylated by the ST3N activity, fetuin was desialylated by mild acid hydrolysis, and incubated with CMP-[^{14}C]Neu5Ac and the recombinant yeast cells. Sodium azide was included in the reaction mixture to block intracellular protein transport to the cell wall or medium. Samples were removed at different times, the cells were pelleted and the supernatants subjected to precipitation with trichloro acetic acid (TCA). Scintillation counting showed that the precipitated radioactivity increased with time (Fig. 2A, closed circles). The precipitated radioactivity was confirmed by mass spectrometry to be fetuin with covalently linked [^{14}C]Neu5Ac residues (see below). Very little radioactivity remained cell-associated, showing that the sialylated protein and CMP-[^{14}C]-Neu5Ac did not adhere to the cells. When completely sialylated fetuin was incubated with the yeast cells (Fig. 2A, open squares), or when asialofetuin was incubated with the parental yeast cells lacking the HSP150Δ-ST3N$_e$ gene (Fig. 2A, open circles), no radioactivity could be precipitated. Authentic fetuin has, in addition to N-glycans, two O-glycans which have two Neu5Ac residues each (Spiro, 1973). Sialylation by Hsp150Δ-ST3N$_e$ was specific for N-glycans, since no [^{14}C]Neu5Ac could be transferred to asialomucin, which carries only O-glycans (Fig. 2A, diamonds).

The sialylation of asialofetuin was enhanced when the incubation with the recombinant yeast cells was carried out in the presence of dithiotreitol (DTT) (Fig. 2B, closed squares). DTT apparently increases the porosity of the cell wall of *S. cerevisiae* by reducing disulphide bridges, thus increased efficiency of sialylation was likely to result from enhanced penetration of asialofetuin into the yeast cell wall. Next we allowed Hsp150Δ-ST3N$_e$ to be synthesized and transported to the cell wall during the assay by omitting sodium azide and adding glucose to the reaction mixture. This resulted in doubling of [^{14}C]-sialylation of asialofetuin (Fig. 2B, open circles). When the recombinant yeast cells

were incubated in the absence of sodium azide with both DTT and glucose, [^{14}C]-sialylation of asialofetuin was further increased (Fig. 2B, open squares). Also larger proteins like bovine prothrombin (M_w 73.6 kD) and human transferrin (M_w 79.6 kD) could be [^{14}C]-sialylated, after removal of their Neu5Ac residues, by the recombinant yeast cells (Table 1). No radioactivity could be TCA-precipitated when the completely sialylated forms of these proteins were used as the acceptor substrate (Table 1).

Efficiency of sialylation. Next we quantitated the degree of sialylation of the asialoglycoproteins. Two of the three N-glycans of fetuin are triantennary and one is biantennary, and thus asialofetuin has 8 terminal galactose residues (Spiro *et al.*, 1973; Rice *et al.*, 1990). Asialofetuin and saturating concentrations of unlabeled CMP-Neu5Ac were incubated for 4 h in the presence of NaN_3 with the recombinant yeast cells expressing Hsp150Δ-ST3N$_e$. The cells were removed by pelleting, and the N-glycans were released by N-glycosidase F digestion and analysed by MALDI-TOF mass spectrometry (Fig. 3). In 4 hours, 31.7% of the terminal galactose residues were sialylated (Fig. 2B). When a parallel cell suspension was pelleted after the 4 h incubation, and the supernatant incubated for another 4 h with a fresh batch of yeast cells and CMP-Neu5Ac (Fig. 3C), 55.3% of the galactose residues were sialylated, demonstrating that both completely and incompletely desialylated protein bound N-glycans could be sialylated. When the incubation was prolonged to 16 h, Neu5Ac was bound to 61.3% of the terminal galactose residues (not shown). A similar overnight incubation of asialotransferrin resulted in sialylation of 41.5% of the exposed galactose residues. To ensure that desialylation of fetuin, prothrombin and transferrin by mild acid hydrolysis had not denatured the proteins, we subjected them to circular dichroism spectroscopy. The spectra of the sialylated and desialylated preparations were superimposable (data not shown).

Kinetic properties of Hsp150Δ-ST3N$_e$. Finally we compared the kinetic properties of yeast cell wall Hsp150Δ-ST3N$_e$ and recombinant ST3N$_e$ produced in insect cells. The K_m values for asialofetuin, lacto-N-tetraose and N-acetyllac-

tosamine were similar (Table 2A). The relative ratios V_{max}/K_m demonstrated that both enzyme preparations preferred lacto-N-tetraose (type 1: Galβ1-3GlcNAc) over N-acetyllactosamine and asialofetuin (type 2: Galβ1-4GlcNAc) (Table 2B). The K_m value of Hsp150Δ-$ST3N_e$ for CMP-Neu5Ac was similar to those reported by others for recombinant $ST3N_e$ from insect cells and authentic isolated rat liver ST3N (Table 2B). A one liter overnight culture containing 18 g (dry weight) of yeast cells contained 117 mU of ST3N activity (lacto-N-tetraose as acceptor).

According to the data presented here, whole living *Saccharomyces cerevisiae* cells expressing the catalytic ectodomain of rat ST3N as a Hsp150Δ-$ST3N_e$ fusion protein in the porous cell wall provide a convenient and inexpensive source of the transferase. The transferase needs not to be purified for use, and neither has the sialylated protein product to be separated from the transferase. Purified transferases have a limited lifetime, whereafter activity is lost, whereas our recombinant yeast cells provide a self-perpetuating source of the enzyme.

Table 1. Transfer of [^{14}C]Neu5Ac to prothrombin and transferrin. The sialylation assay was performed in the presence of NaN_3 as described in the Legend of Figure 1.

Time (h)	Protein-bound [^{14}C]Neu5Ac (cpm)			
	Prothrombin	Asialoprothrombin	Transferrin	Asialotransferrin
2	292	3856	428	994
4	657	9016	341	1885

Table 2. Kinetic parameters of various ST3N preparations. The K_m values and the relative V_{max}/K_m values are given for the indicated acceptor substrates. (The highest value for V_{max}/K_m is 100). In **(A)** CMP-Neu5Ac was available in saturating concentrations, and the protein or sugar substrate in varying concentrations. In **(B)**, lacto-N-tetraose was available in saturating concentrations, and varying concentrations of CMP-Neu5Ac up to 10 mM were mixed with a constant amount of CMP-[^{14}C]Neu5Ac. Enzyme preparations: Hsp150Δ-ST3N$_e$ in the wall of yeast cells; recombinant ST3N$_e$ from insect cells (rST3N$_e$); authentic ST3N from rat liver.

(A)

	Asialofetuin		Lacto-N-tetraose		N-acetyllactosamine	
	K_m (μM)	V_{max}/K_m	K_m (μM)	V_{max}/K_m	K_m (μM)	V_{max}/K_m
Hsp150Δ-ST3N$_e$	42.7	5.8	51.00	100	717.7	5.9
rST3N$_e$	34.4	10.9	43.0	100	1014	11.3

(B)

	K_m (μM) for CMP-Neu5Ac	Reference
Hsp150Δ-ST3N$_e$	55.0	this work
rST3N$_e$	74.1	Williams *et al.*, 1995
ST3N	57.3	Gross *et al.*, 1989

References

Andersen, D.C. and Goochee, C.F. (1994). Curr. Opin. Biol. 5:546–549.

Börnsen, K.O., Mohr, M.D. and Widmer, H.M. (1995). Rapid Commun. Mass Spectrom. 9:1031–1034.

Curling, E.M.A., Hayter, P.M., Baines, A.J., Bull, A.T., Gull, K., Strange, P.G. and Jenkins, N. (1990). Biochem. J. 272:333–337.

Field, M.C. and Wainwright, L.J. (1995). Glycobiology 5:463–472.

Fukuda, M.N., Sasaki, H., Lopez, L. and Fukuda, M. (1989) Blood 73:84–89.

Goochee, C.F., Gramer, M.J., Andersen, D.C., Bahr, J.B. and Rasmussen, J.R. (1991) Bio/Technology 9:1348–1355.

Gramer, M.J., Goochee, C.F., Chock, V.Y., Brousseau, D.T. and Sliwkowski, M.B. (1995) Bio/Technology 13:692–698.

Gross, H.J., Krause, J.M., Paulson, J.C., Feeney, R.E. and Brossmer, R. (1989). Biochemistry 28:7386–7392.

James, D.C., Freedman, R.B., Hoare, M., Ogonah, O.W., Rooney, B.C., Larionov, O.A., Dobrovolsky, V.N., Lagutin, O.V. and Jenkins, N. (1995). Bio/Technology 13:592–596.

Krezdorn, C.H., Kleene, R.B., Watzele, M., Ivanov, S.X., Hokke, C.H., Kamerling, J.P. and Berger, E. (1994). Eur. J. Biochem. 220:809–817.

Lee, E.U., Roth, J. and Paulson, J.C. (1989). J. Biol. Chem. 264:13848–13855.

Maiorella, B.L., Winkelhake, J., Young, H., Moyer, B., Bauer, R., Hora, M., Andya, J., Thomson, J., Patel, T. and Parekh, R. (1993). Bio/Technology 11: 387–392.

Makarow, US patent No. 5,677,172.

Mattila, P., Joutsjoki, V., Kaitera, E., Majuri, M.–L., Niittymäki, J., Saris, N., Maaheimo, H., Renkonen, O., Renkonen, R. and Makarow, M. (1996). Glycobiology 6:8:851–859.

Nyman, T.A., Kalkkinen, N., Tölö, H. and Helin, J. (1998). Eur. J. Biochem. 253:485–493.

Papac, D.I., Wong, A. and Jones, A.J.S. (1996). Anal. Chem. 68:3215–3223.

Patel, T.P., Parekh, R.B., Moellering, B.J. and Prior, C.P. (1992). Biochem. J. 285:839-845.

Renkonen, O., Niemelä, R., Leppänen, A., Maaheimo, H., Seppo, A., Penttilä, L. and Vikman, A. (1991). Glycoconjugate J. 8:368-375.

Rice, K.G., Rao, N.B.N. and Lee, Y.C. (1990). Anal. Biochem. 184:249-258.

Russo, P., Kalkkinen, N., Sareneva, H., Paakkola, J. and Makarow, M. (1992). Proc. Natl. Acad. Sci. USA 89:3671-3675.

Sareneva, T., Cantell, K., Pyhälä, L., Pirhonen, J. and Julkunen, I. (1993). J. Interferon Res. 13:267-269.

Schreuder, M.P., Brekelmans, S., van den Ende, H. and Klis, F.M. (1993). YEAST 9:399-409.

Simonen, M., Jämsä, E. and Makarow, M. (1994). J. Biol. Chem. 269:19: 13887-13892.

Simonen, M., Vihinen, H., Jämsä, E., Arumäe, U., Kalkkinen, N. and Makarow, M. (1996). YEAST 12:457-466.

Spiro, R.G. (1960). J. Biol. Chem. 235:10:2860-2869.

Spiro, R.G. (1973). Adv. Protein Chem. 27:349-467.

Szkudlinski, M.W., Thotakura N.R., Bucci, I., Joshi, L.R., Tsai, A., East-Palmer, J., Shiloah, J. and Weintraub, B.D. (1993). Endocrinology 133:1490-1503.

Williams, M.A., Kitagawa, H., Datta, A.K., Paulson, J.C. and Jamieson, J.C. (1995). Glycoconjugate J. 12:755-761.

Claims

1. A process for sialylating glycoproteins, comprising incubating said proteins with recombinant yeast cells expressing a sialyltransferase enzyme or a fragment thereof as a fusion protein with Hsp150Δ carrier.

2. The process according to claim 1, wherein the recombinant yeast cells express the catalytic ectodomain of rat liver α2,3-sialyltransferase ($ST3N_e$).

3. The process according to claim 1, wherein the transformed yeast cells are *Saccharomyces* cells.

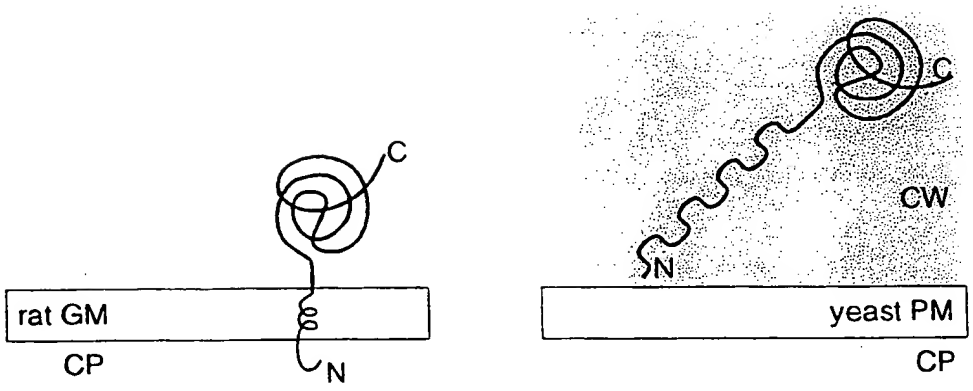
4. The process according to claim 3, wherein the transformed yeast cells are *Saccharomyces cerevisiae* cells.

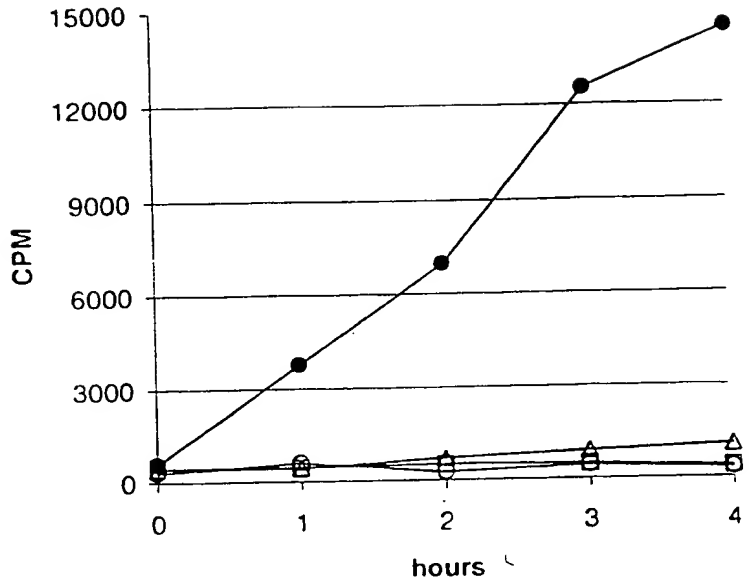
5. The process according to claim 1, wherein the incubation is carried out in the presence of dithiotreitol.

6. The process according to claim 1, wherein the incubation is carried out in the presence of glucose.

7. The process according to claim 1, wherein the incubation is carried out in the presence of dithiotreitol and glucose.

Fig. 1

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12000
9000
6000
3000
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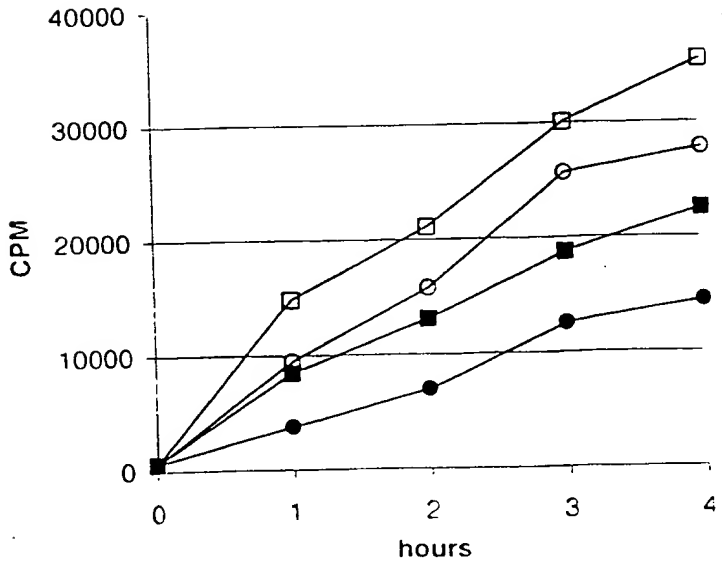
CPM
0
1
2
3
4
hours

Fig. 2A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CPM
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2
3
4
hours

Fig. 2B

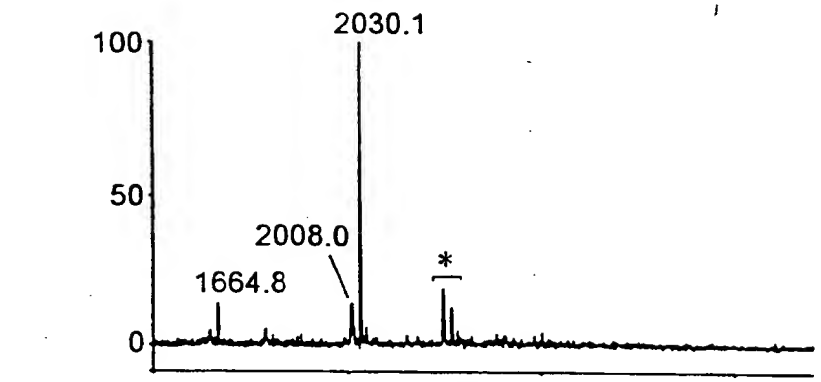

Fig. 3A

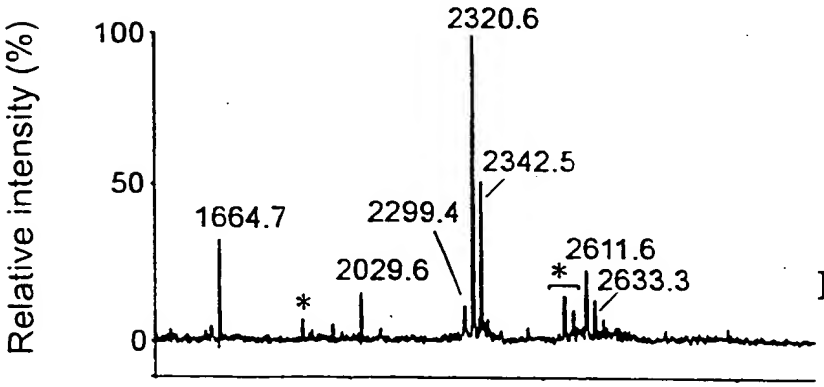

Fig. 3B

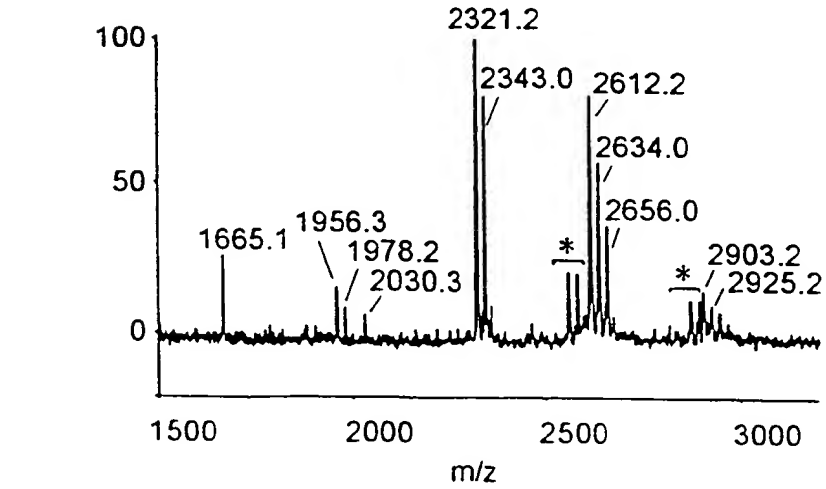

Fig. 3C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 99/00410

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12P 21/00 // C12P 19/26, C12N 15/62, C12P 15/81
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Glycobiology, Volume 6, No 8, 1996, pirkko Mattila et al, "Targeting of active rat alpha2,3-sialyltransferase to the yeast cell wall by the aid of the hsp 150 -carrier: toward synthesis of sLeX-decorated L-selectin ligands", page 851 - page 859, see title, and the whole document -- --------	1-7

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 Sept 1999	07 -09- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Patrick Andersson/EÖ Telephone No. + 46 8 782 25 00

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Published
With international search report.

(54) Title: PROCESS FOR SIALYLATING GLYCOPROTEINS

(57) Abstract

The present invention relates to genetic engineering, and provides a novel process for sialylating glycoproteins. In the process said proteins are incubated with recombinant yeast cells expressing a sialyltransferase enzyme as a fusion protein with Hsp150Δ carrier in the porous cell wall.

*(Referred to in PCT Gazette No. 5/2000, Section II)

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